



**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	LI	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TG	Togo
DE	Germany	MG	Madagascar	UA	Ukraine
DK	Denmark	ML	Mali	US	United States of America
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

## ELECTROCHEMICAL DETECTION OF DNA HYBRIDISATION

This invention relates to electrochemical detection of DNA hybridisation. The invention also relates to apparatus for detection of DNA hybridisation and to the use of the method for detection of genetic defects.

DNA analysis is attaining increased importance in the diagnosis of disease. Over 4000 single gene defects have been implicated in the pathology of human inherited disease. Diagnosis of several genetic diseases can be carried out using DNA technology. Commonly used methods include restriction fragment length polymorphism (RFLP) analysis, polymerase chain reaction (PCR) techniques and gene probe/hybridisation analysis. Applicability of any one of these techniques to diagnosis depends to a large extent on the complexity of the genetic defect in question. For diseases where the precise genetic defect is known and proves to be a simple mutation (for example in sickle cell anaemia and phenylketonuria), both PCR and direct hybridisation techniques can be applied. PCR diagnosis generally involves the amplification reaction (PCR) followed by a gel electrophoresis step. Hybridisation analysis of DNA without any need for electrophoresis, or immediately following PCR amplification would provide more rapid analysis time thereby hastening diagnosis.

The use of oligonucleotide probes to detect the presence of specific genes involves a procedure called hybridisation. Under appropriate conditions of pH, temperature and ionic strength single stranded DNA fragments will hydrogen bond or hybridise with complementary single stranded pieces of DNA. The stability of the double stranded complex or hybrid, depends upon the degree of complementarity between the two nucleic acid strands. By increasing the pH or temperature, or by lowering the ionic strength, the conditions of hybridisation may be made more or less stringent. Under more stringent conditions, the two strands of an imperfectly matched hybrid will dissociate whereas two more perfectly complementary strands will remain in duplex form.

In practice, the test DNA is first denatured (rendered single stranded) with heat and is mixed with the probe. Under appropriate conditions the nucleic acid probe is then allowed to hybridise with any complementary sequences which might be present in the test DNA. The prototype of this method is the widely used Southern blot technique.

Hybridising of fragments within the test DNA may be detected by a variety of methods. The most common method is that of labelling the probe with a radioactive phosphorus. Nick translation is a commonly used  $^{32}\text{P}$  labelling procedure. In this method unlabelled nucleotides in the DNA probe are replaced by labelled ones. The sequence of the probe is not changed and probes with specific activities of the order of  $10^8$  to  $10^9$  cpm/ $\mu\text{g}$  of DNA may be generated allowing a detection of  $<100\text{pg}$  of target sequence (about  $10^{-2}\text{fmol}$  of a 10kb DNA fragment). Other  $^{32}\text{P}$  labelling procedures have been devised.

Although radiolabelled probes allow the sensitive detection of complementary sequences there are some notable disadvantages. The radiation hazard associated with  $^{32}\text{P}$  requires that laboratory personnel are appropriately shielded and monitored. Provision must also be made for radioactive waste disposal. In addition, the short half-life of  $^{32}\text{P}$  (14-31 days) requires that fresh probe have to be prepared at frequent intervals.

Non-radioactive methods have been introduced to circumvent problems associated with radioactivity, for example, enzymatic incorporation of biotinylated nucleotides or digoxigenated(DIG)-nucleotides into a probe. Probes in a stable hybrid are then detected by means of avidin or streptavidin for biotin or anti-DIG antibody labelled with a fluorophore or enzymes such as horse-radish peroxidase or alkaline phosphatase. The detection sensitivity of these methods is comparable to  $^{32}\text{P}$  labelled probes. Another non-radioactive method has involved direct coupling of enzymes to single stranded DNA probes followed by colorimetric detection after hybridisation. Incorporation of fluorophores into synthetic probes and the use of chelated rare earth metals

allow detection of hybridisation by fluorimetry and time resolved fluorimetry respectively.

When analysis is carried out by current methods of DNA probe hybridisation, a result is generally not achievable in less than six days. A Southern blot analysis of genomic DNA may take five to ten days. Time consuming steps in the procedure include DNA extraction, restriction enzyme digestion and gel electrophoresis, transfer and hybridisation. Each of these steps requires about one day to complete, in addition, to from one to seven days for auto-radiographic exposure of the hybridised blot. Combined PCR and hybridisation procedures may be performed in approximately 2 hours, but this must be followed by the time required for detection of the labelling system in use. There is a need not only for the development of non-radioactive detection methods but also for more rapid analysis if nucleic acid hybridisation is to become a routine procedure.

Studies of the properties of DNA using polarographic (voltammetric) methods have achieved considerable progress. The electroactive groups in nucleotides are the bases, while deoxyribose and phosphate groups are polarographically non-reducible. The electrochemistry of DNA was initially investigated by the technique of linear sweep (ls) polarography in which a dropping mercury electrode (DME) was polarised with a linearly changing voltage. With this technique thermally denatured DNA, which is single stranded (ssDNA), was observed to give a reduction current in the presence of 300mM ammonium formate at neutral pH, whereas double stranded DNA (dsDNA) was inactive under the same conditions. The difference in behaviour between dsDNA and ssDNA was most likely due to the accessibility of the bases in the two different forms.

Ls polarography is an insensitive technique for DNA investigations. Better results have been obtained using differential pulse polarography (dpp). Both ssDNA and dsDNA give a well defined peak but at widely different potentials. For calf thymus DNA, ssDNA (30µg/ml) yields a peak at about -1.4V while a much smaller peak is observed with dsDNA

(350- $\mu$ g/ml) at about -1.3V.

The techniques of adsorptive stripping voltammetry (AdSV) and adsorptive transfer stripping voltammetry (AdTSV) have resulted in a large increase in the sensitivity of electrochemical analysis of nucleic acids, with potential detection limits of subnanogram quantities of DNA.

According to a first aspect of the present invention there is provided a method of detecting DNA hybridisation which includes the steps of:

- denaturing a specimen of DNA;
- contacting the specimen with an electrochemical electrode;
- measuring a reference polarographic signal characteristic of ssDNA;
- contacting said sample with an oligonucleotide probe under conditions where hybridisation may occur;
- removing any excess reagents;
- obtaining a polarographic signal from said electrode;
- and,
- comparing said signal with the reference signal.

Electrochemical DNA biosensors have wide application in DNA diagnostics, particularly in routine nucleic acid sequence detection procedures that are accurate, sensitive, rapid and economical. Biosensors in accordance with the present invention not only eliminate use of radio isotopes but also avoid the need for time consuming detection steps. Electrophoresis and transfer of digested DNA to nitrocellulose is not required. Immobilised probes may be employed so that minimal sample preparation is necessary.

The polarographic signal is preferably obtained by square wave voltammetry, square wave adsorptive stripping voltammetry and square wave adsorptive transfer stripping voltammetry.

The oligonucleotide probe may be provided in solution. In preferred embodiments of the invention the oligonucleotide probe is immobilised on or adjacent the electrode. Carbodiimide mediated end attachment of DNA to cellulose is an appropriate method whereby immobilised probes may be obtained.

This method has the advantage that screen printed electrodes can be produced in large volumes at low cost. Alternative methods of immobilisation may use such solid supports as Sephadex, Sephacryl, Sephadex and controlled pore glass. Quantification of the immobilised DNA may be carried out using the nuclease assay described by Bunemann (Nucl. Acids Res. 10, 7163 to 7180). Platinum or other conventional electrodes may be used in accordance with this invention. More preferably carbon electrodes may be used as these facilitate manufacture by screen printing. Preferred carbon electrodes include glassy carbon, pyrolytic graphite and wax impregnated spectroscopic graphite.

According to a second aspect of the present invention a DNA hybridisation detection system includes a support carrying a carbon electrode and an immobilised oligonucleotide probe located on or adjacent said electrode.

According to a third aspect of the present invention there is provided use of electrochemical detection of DNA hybridisation for detection of a genetic defect in a specimen of DNA.

Particular defects include physical disorders associated with Beta-globin (sickle cell anaemia), tyrosinase, phenylalanine hydroxylase (phenylketonuria) and adenosine deaminase (combined immunodeficiency).

A preferred method involves the steps of:

- isolation of test DNA;
- digestion with restriction endonuclease to form DNA of appropriate sizes;
- denaturation and hybridisation with oligonucleotide probe immobilised on an electrode;
- washing to remove unbound nucleic acid;
- electrochemical measurement of the immobilised probe before and after hybridisation.

The method may comprise the step of washing to remove unbound sample nucleic acid.

The method is further described by means of example but

not in any limitative sense with reference to the accompanying drawings of which:

Figure 1 is a voltammogram for native and heat denatured calf thymus DNA.;

Figure 2 is a DNA calibration curve using OSWV; and

Figure 3 is a scheme illustrating the present invention.

Figures 1 and 2 illustrate experimental results obtained with a glassy carbon electrode. Osteryoung square wave voltammograms for (A) native and (B) heat denatured calf thymus DNA were measured at a DNA concentration of  $600 \mu\text{gcm}^{-3}$  in 0.5M sodium acetate buffer at pH 5.0. Peaks 1 and 2 shown in Figure 1 correspond to oxidation of guanine and adenine respectively. The step potential was 4mV, the amplitude was 25mV and the frequency was 12Hz.

Figure 2 is a DNA calibration curve obtained using Osteryoung square wave voltammetry. Curve A is the current obtained at peak 1 of Figure 1 and curve B is the peak 2 current. Measurements were carried out in 0.5M sodium acetate buffer at pH 5.0. The step potential was 4mV, the amplitude 25mV and frequency 250Hz.

Figure 3 is a scheme illustrating use of the present invention. An electrode with a specific immobilised DNA probe 1 is contacted with a DNA sample to be analyzed. The sample may be amplified by PCR if necessary. An initial reading 2 from the electrode shows presence of peaks 1 and 2 as shown in Figure 1. Incubation of the electrode and DNA sample under stringent hybridisation conditions will result in hybridisation of the immobilised DNA on the probe with any complementary strands present in the sample. If no hybridisation occurs the second electrochemical reading 3 will be similar to the initial reading 2. This would demonstrate the absence of the genetic defect characterised by the DNA immobilised on the probe. Occurrence of hybridisation removes or substantially reduces peaks 1 and 2 from the second electrochemical reading as shown in 4. This demonstrates hybridisation of the immobilised DNA showing the presence of the genetic defect.



CLAIMS:

1. A method of detecting DNA hybridisation including the steps of:
  - denaturing a specimen of DNA;
  - contacting the specimen with an electrochemical electrode;
  - measuring a reference polarographic signal characteristic of ssDNA;
  - contacting said sample with an oligonucleotide probe under conditions where hybridisation may occur;
  - removing any excess reagents;
  - obtaining a polarographic signal from said electrode;and,
  - comparing said signal with the reference signal.
2. A method of detecting DNA hybridisation as claimed in claim 1, wherein the polarographic signal is obtained by square wave voltammetry, square wave adsorptive stripping voltammetry, or square wave adsorptive transfer stripping voltammetry.
3. A method of detecting DNA hybridisation as claimed in claim 1 or claim 2, wherein the oligonucleotide probe is provided in solution.
4. A method of detecting DNA hybridisation as claimed in any of claims 1 to 3, wherein the oligonucleotide probe is immobilised on the electrode.
5. A method of detecting DNA hybridisation as claimed in any of claims 1 to 4, wherein the oligonucleotide probe is immobilised adjacent the electrode.
6. A method of detecting DNA hybridisation as claimed in any of claims 1 to 5, wherein immobilised probes are obtained by carbodiimide mediated end attachment of DNA to cellulose.

7. A method of detecting DNA hybridisation as claimed in any one of claims 1 to 6, wherein the electrodes are composed of glassy carbon, pyrolytic graphite, or wax impregnated spectroscopic graphite.

8. A DNA hybridisation detection system which includes a support; a carbon electrode and an immobilised oligonucleotide probe being located adjacent said electrode.

9. A method of electrochemical detection of DNA hybridisation for detection of a genetic defect in a specimen of DNA which includes the steps of:

isolation of test DNA;

digestion with restriction endonuclease to form DNA of appropriate sizes;

denaturation and hybridisation with oligonucleotide probe immobilised on an electrode;

washing to remove unbound nucleic acid;

electrochemical measurement of the immobilised probe before and after hybridisation.

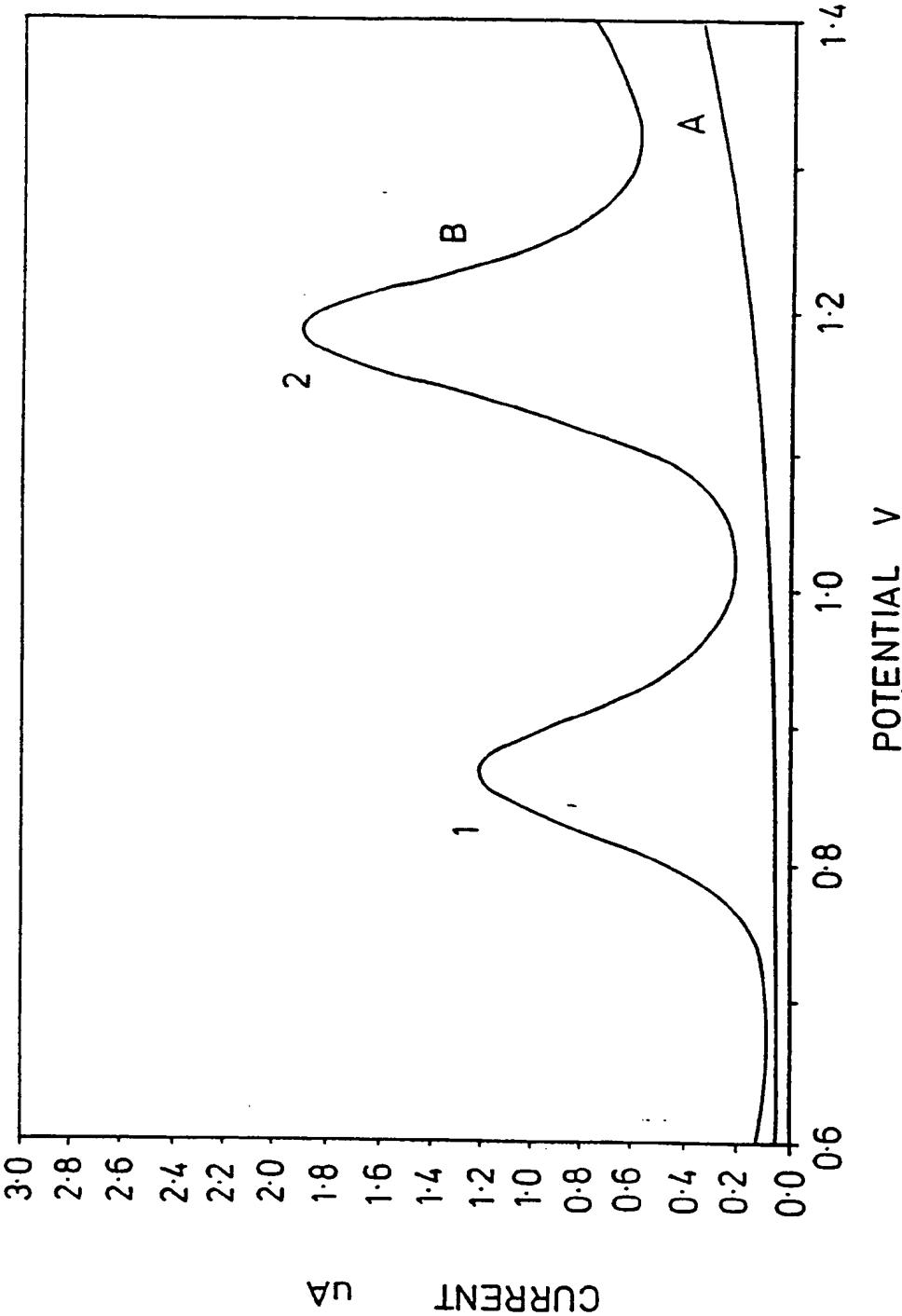


FIG. 1

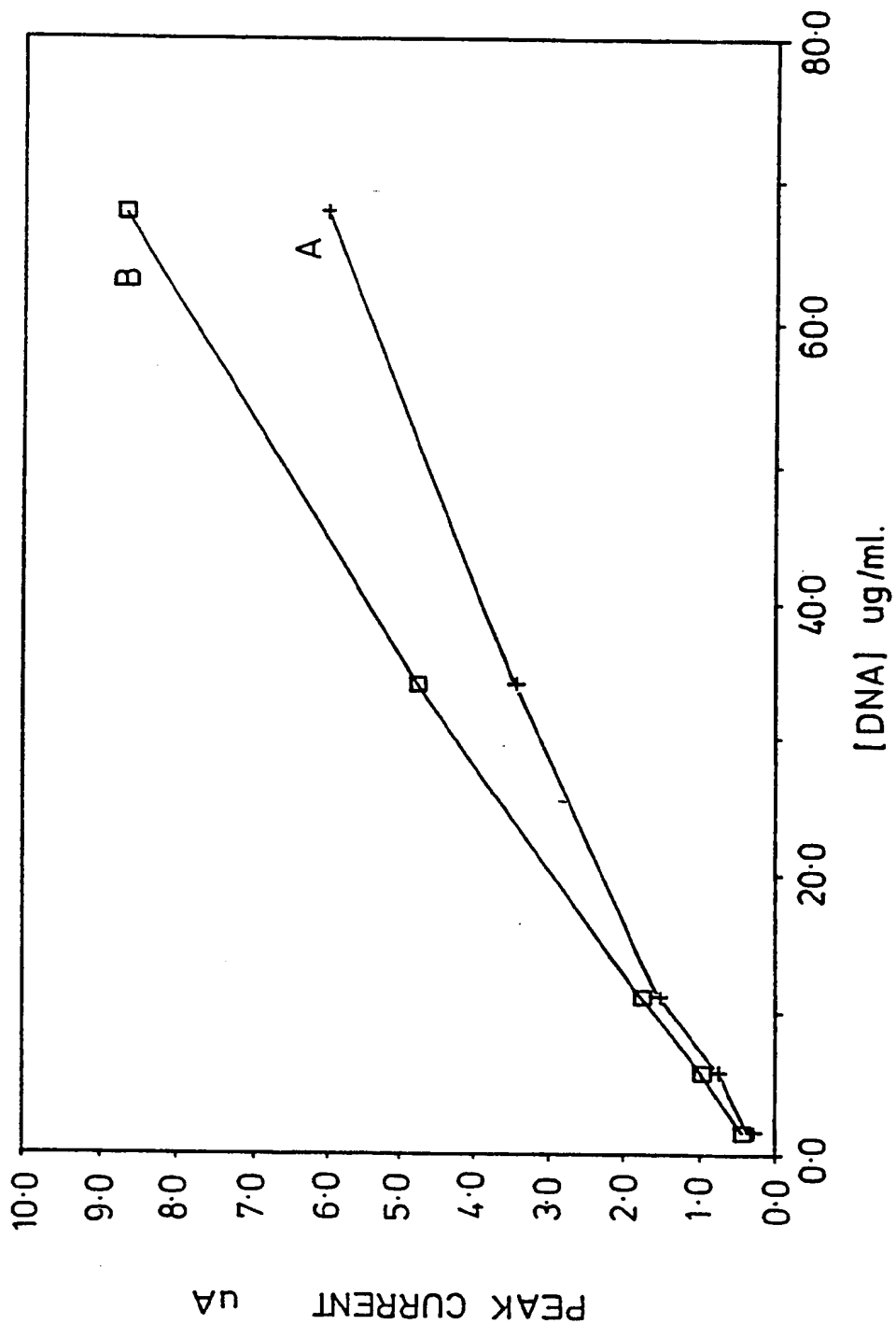


FIG. 2

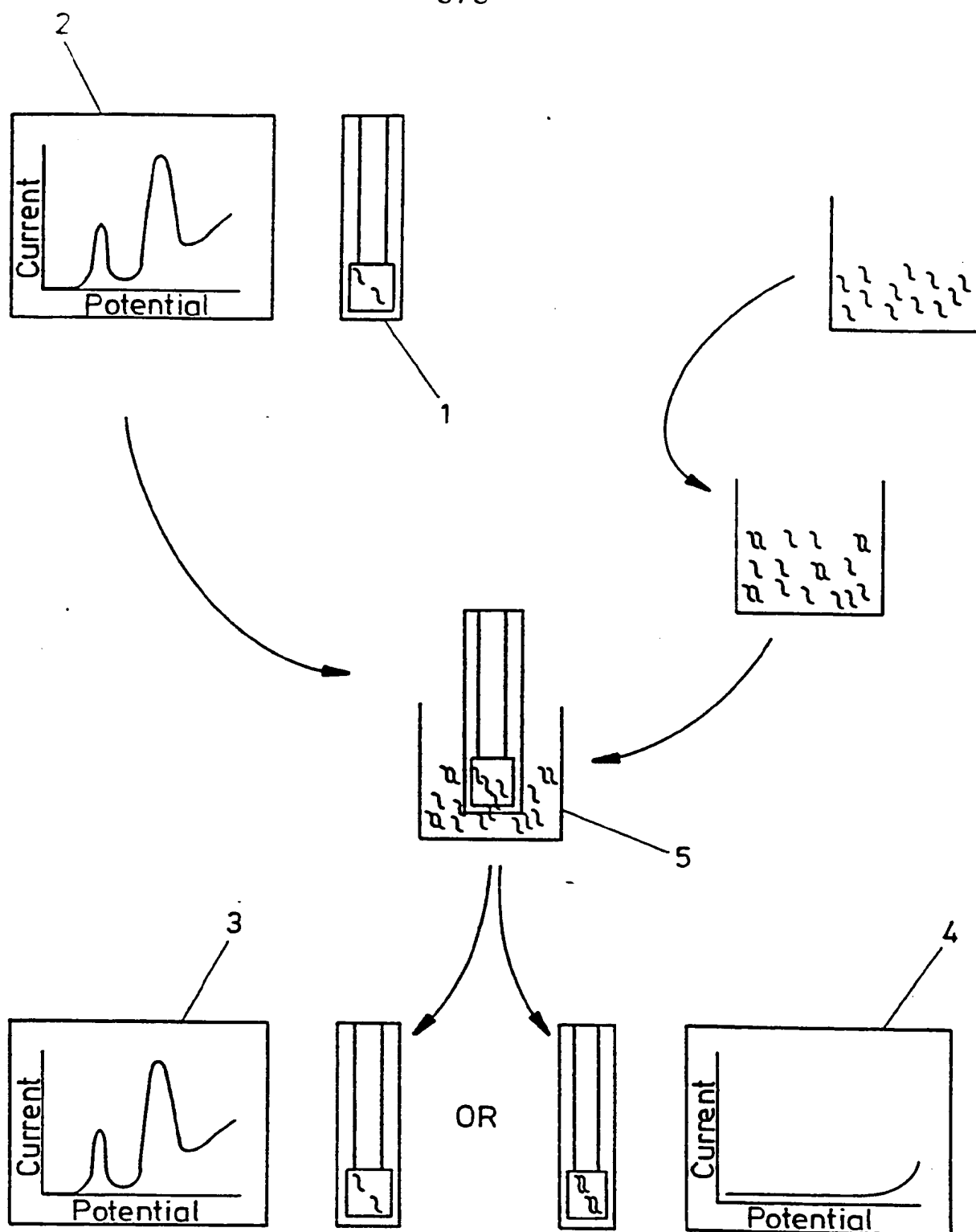


FIG. 3

SUBSTITUTIVE SHEET

# INTERNATIONAL SEARCH REPORT

International Publication No

PCT/GB 93/00631

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all)* According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl. 5 C12Q1/68;                      G01N27/00																				
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border: 1px solid black; padding: 2px;">Minimum Documentation Searched?</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 30%; border: 1px solid black; padding: 2px;">Classification System</td> <td style="border: 1px solid black; padding: 2px;">Classification Symbols</td> </tr> <tr> <td style="border: 1px solid black; padding: 2px;">Int.Cl. 5</td> <td style="border: 1px solid black; padding: 2px;">C12Q</td> </tr> </table> <div style="text-align: center; border: 1px solid black; padding: 2px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched<sup>8</sup></div>			Classification System	Classification Symbols	Int.Cl. 5	C12Q														
Classification System	Classification Symbols																			
Int.Cl. 5	C12Q																			
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; padding: 2px;">Category<sup>10</sup></th> <th style="width: 70%; padding: 2px;">Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 20%; padding: 2px;">Relevant to Claims No.<sup>13</sup></th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">           CHEMICAL ABSTRACTS, vol. 94, no. 23,            1981, Columbus, Ohio, US;            abstract no. 187973a,            page 271 ;            see abstract            &amp; STUD. BIOPHYS., 82(1), 17-26 PY - 1981            Brabec, V. 'Voltammetry of biosynthetic            polynucleotides at carbon'            ---         </td> <td style="text-align: center; vertical-align: top; padding: 5px;">1,2,7</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">           BIOELECTROCHEM. BIOENERG., 8(4), 437-49            1981,            BRABEC, VIKTOR 'Nucleic acid analysis by            voltammetry at carbon electrodes'            see the whole document            ---         </td> <td style="text-align: center; vertical-align: top; padding: 5px;">8</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">           see the whole document            ---         </td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-7</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">           DE,A,3 513 168 (T. DANDEKAR)            16 October 1986            see the whole document            ---         </td> <td style="text-align: center; vertical-align: top; padding: 5px;">8</td> </tr> <tr> <td colspan="3" style="text-align: center; padding: 5px;">-/-</td> </tr> </tbody> </table>			Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claims No. <sup>13</sup>	X	CHEMICAL ABSTRACTS, vol. 94, no. 23, 1981, Columbus, Ohio, US; abstract no. 187973a, page 271 ; see abstract & STUD. BIOPHYS., 82(1), 17-26 PY - 1981 Brabec, V. 'Voltammetry of biosynthetic polynucleotides at carbon' ---	1,2,7	X	BIOELECTROCHEM. BIOENERG., 8(4), 437-49 1981, BRABEC, VIKTOR 'Nucleic acid analysis by voltammetry at carbon electrodes' see the whole document ---	8	A	see the whole document ---	1-7	X	DE,A,3 513 168 (T. DANDEKAR) 16 October 1986 see the whole document ---	8	-/-		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claims No. <sup>13</sup>																		
X	CHEMICAL ABSTRACTS, vol. 94, no. 23, 1981, Columbus, Ohio, US; abstract no. 187973a, page 271 ; see abstract & STUD. BIOPHYS., 82(1), 17-26 PY - 1981 Brabec, V. 'Voltammetry of biosynthetic polynucleotides at carbon' ---	1,2,7																		
X	BIOELECTROCHEM. BIOENERG., 8(4), 437-49 1981, BRABEC, VIKTOR 'Nucleic acid analysis by voltammetry at carbon electrodes' see the whole document ---	8																		
A	see the whole document ---	1-7																		
X	DE,A,3 513 168 (T. DANDEKAR) 16 October 1986 see the whole document ---	8																		
-/-																				
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>10</sup> Special categories of cited documents : <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"A" document member of the same patent family</p> </div> </div>																				
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border: 1px solid black; padding: 5px;">           Date of the Actual Completion of the International Search  <div style="text-align: center; font-weight: bold;">08 JULY 1993</div> </td> <td style="width: 50%; border: 1px solid black; padding: 5px;">           Date of Mailing of this International Search Report  <div style="text-align: center; font-weight: bold;">30. 07. 93</div> </td> </tr> <tr> <td style="border: 1px solid black; padding: 5px;">           International Searching Authority  <div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div> </td> <td style="border: 1px solid black; padding: 5px;">           Signature of Authorized Officer  <div style="text-align: center; font-weight: bold;">MOLINA GALAN E.</div> </td> </tr> </table>			Date of the Actual Completion of the International Search <div style="text-align: center; font-weight: bold;">08 JULY 1993</div>	Date of Mailing of this International Search Report <div style="text-align: center; font-weight: bold;">30. 07. 93</div>	International Searching Authority <div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center; font-weight: bold;">MOLINA GALAN E.</div>														
Date of the Actual Completion of the International Search <div style="text-align: center; font-weight: bold;">08 JULY 1993</div>	Date of Mailing of this International Search Report <div style="text-align: center; font-weight: bold;">30. 07. 93</div>																			
International Searching Authority <div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center; font-weight: bold;">MOLINA GALAN E.</div>																			

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	CHEMICAL ABSTRACTS, vol. 89, no. 1, 1978, Columbus, Ohio, US; abstract no. 1823v, page 174 ; see abstract & BULL. CHEM. SOC. JPN., 51(4), 1235-6 PY - 1978 Yao, Toshio; Wasa, Tamotsu; Musha, Soichiro 'The anodic voltammetry of deoxyribonucleic acid at a glassy carbon electrode' -----	1,2,7
A	WO,A,8 702 066 (UNIVERSITY OF SOUTHERN MISSISSIPPI) 9 April 1987 -----	-
A	EP,A,0 149 339 (GENETICS INTERNATIONAL) 24 July 1985 -----	-

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

GB 9300631  
SA 72117

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

08/07/9

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE-A-3513168	16-10-86	US-A- 4777019	11-10-88
WO-A-8702066	09-04-87	AU-A- 6404286	24-04-87
		EP-A, B 0239613	07-10-87
		JP-T- 63501920	04-08-88
EP-A-0149339	24-07-85	AU-B- 569076	21-01-88
		AU-A- 2775384	08-11-84
		AU-B- 583258	27-04-89
		AU-A- 3832985	26-06-85
		CA-A- 1220818	21-04-87
		CA-A- 1223639	30-06-87
		EP-A- 0125139	14-11-84
		WO-A- 8502627	20-06-85
		JP-T- 61500706	17-04-86
		US-A- 4840893	20-06-89